IDENTIFICATION OF ANTHOCYANIDIN PIGMENTS IN THREE VARIETIES OF PELARGONIUM HORTORUM BY CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS

by

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B. Sc. (Ag), Osmania University, Hyderabad, India, 1953

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1961

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INTRODUCTION

The anthocyanins are responsible for the blue, purple and red colors in many flowers. Although this fact was known for many years, it has been only during the comparatively recent times that the specific anthocyanins and anthocyanidins responsible for the color in flower petals of some of the important ornamental flowers were identified. Very often more than one anthocyanin was present in a particular plant species and this made the identification of anthocyanins and anthocyanidins very difficult. The application of paper chromatography in the identification of anthocyanidins is a very recent technique which has proved useful in the identification and separation of anthocyanidins. The presence of a sugar moiety in anthocyanins often presents considerable difficulty in the identification and separation of anthocyanidins. To identify the anthocyanidins, the sugar moiety must first be removed by acid hydrolysis. The sugar, which can be of more than one type, not only affects the color but also makes the separation of the pigments difficult.

It is rather surprising that though some work has been done on the identification and isolation of anthocyanidins in Euphorbia (2) and Tulip (25), the common horticultural geranium has been neglected as far as the work on the anthocyanidins is concerned.

The purpose of the present investigation was to isolate and identify by chromatographic and spectrophotometric methods, the anthocyanidins in the flower petals of three varieties of common cultivated geranium. (*Pelargonium hortorum*)

REVIEW OF LITERATURE

The innumerable shades of blue, purple, violet, mauve and magenta, and nearly all the red, which appear in the flowers, fruits, leaves and stems are due to anthocyanin pigments. By comparison with other compounds the anthocyanin pigments of plants have received considerable attention. These coloring matters have sometimes been spoken of as water soluble pigments, since they are in a state of solution in the cell sap, as contrasted with those which are in some way bound up with the structure of the organized protoplasmic bodies known as plastids.

The word anthocyanin was first coined by Marquart (49) who used it for the red, violet and blue pigments of flowers. Man's interest in the color pigments, however, started much earlier. Boyle (13) in the year 1664 published the results of his experiments on changes in color on the addition of acids and alkalies to extracts from flowers and other parts of the plant. Malpighi (48) gave an account of the histology of the colored pigments. Grew (24) in a series of lectures given before the Royal Society, London, England, discussed the general considerations on the colors of plants and views as to their origin. Macaire-Princep (47) stated that the red pigments are oxidized chlorophyll. Candolle (18) classified vegetative pigments into two series, xanthic and cyanic. As stated earlier, Marquart (49) was the first to use the word anthocyanins for the red, blue and violet pigments of flowers. He believed that they are formed by the dehydration of chlorophyll. To the yellow pigments of plants he gave the name anthoxanthin. Onslow

(52) states that the term anthocyanin was retained in the same sense to the present day in which sense its coiner had used it, though other rival terms which are now obsolete, such as erythrophyll, cyanophyll and cyanin have been used from time to time.

Weigert (72) by qualitative tests differentiated anthocyanin pigments into two groups. Kruths (42) gave a detailed account of the biological significance of anthocyanins in plants. The morphological and histological distribution of plant pigments was described by Buscalioni and Pollacci (16). Grafe (23) reported the preparation and analysis of anthocyanin pigments in hollyhock (*Althea rosea*).

The fact that anthocyanins are present in plants as glycosides was brought forward by Willstatter and his collaborators (75-100). They said that these pigments belong to a group of glycosides, the sugar free pigments or aglycones of which are called anthocyanidins. Wheldale (74) in the year 1916 published the book "Anthocyanin Pigments of Plants." A revised edition of this book was published by Onslow (52) in the year 1925. The book describes in great detail the morphological and histological distribution of anthocyanins, their isolation and constitution, factors influencing their formation and the genetics of anthocyanins. Willstatter et al (82) in a series of articles published from 1913-1924, did pioneering work on the various aspects of anthocyanins. Investigations by Karrer (34), Robinson (57) and Willstatter and Weil (90) have revealed that anthocyanins are found in chloride form, in the form of eight anthocyanidins. The structural formulas of anthocyanins have been given by them (34) (36). Willstatter et al (82) have pointed out

that the various anthocyanidins are relatives of 2-phenylbenzopyrilium, usually found in the form of its chloride, and as such designated as the flavilium chloride. Willstatter and Mallison (85) state that by treating anthocyanidins with potassium hydroxide, two products, a phenol and a substituted phenol-carbolic acid are obtained. They further add that pelargonidin chloride which is an anthocyanidin, is decomposed into phloroglucinol and p-hydrobenzoic acid. Willstatter and Weil (90) stated that by boiling the anthocyanins for a short time in 20 percent acid the pigments may be split into anthocyanidins and sugar components. Karrer et al (35) (36) point out that oxidative degradation with hydrogen peroxide results in certain anthocyanins in oxidation products which are still closely related to the original anthocyanins.

Robinson and Robinson (54) surveyed the work to 1931. They also published a detailed list of plants indicating the anthocyanin pigments they contain.

Jonesco (33), Rosenheim (61) and Schriner et al (64) state that anthocyanidins have been observed in plants only in rare cases. They further add that as a rule they occur in nature attached to one or more sugars as anthocyanins. Blank (14) is of the opinion that the anthocyanins appearing in nature are partly mono- and partly di-glycosides. Sugars like glucose, rhamnose, galactose and gentiobiose have been isolated as sugar components. One of these sugar molecules is always attached at the 3-position. If a second sugar molecule is present, it is either coupled with the first or attached to the anthocyanidin in the 5-position. Robinson (59) divides the anthocyanins appearing in nature into the following

groups.

1. 3-monoglucosides and 3-monogalactosides
2. 3-rhamnoglucosides and other 3-pentoseglycosides
3. 3-biosides
4. 3-5-diglucosides
5. acylated anthocyanins

Willstatter et al (81) state that 3-5-diglucosides are the most widely distributed in nature; they are also the best known.

Blank (14), Robinson and Robinson (55) and Scott-Moncrieff (67) reported that variation in sugar moiety of the anthocyanin pigment affected color. They found that monoglycosides were redder than the corresponding pentose-glycosides, while 3-biosides and 3-5-diglycosides were bluer.

Blank (14) has reviewed the anthocyanin work done until 1947. While discussing the properties of anthocyanins he states that all anthocyanins are soluble in water, as is shown by the fact that they are present in the cell sap of vacuoles. The anthocyanidins on the other hand are not soluble in water. The glycosides are insoluble in non-hydroxylic solvents such as ether, chloroform and benzene. Molisch (51) discovered the important fact that the anthocyanins in solution produce well crystallizing products when treated with acids. This fact was used by Willstatter et al (100) in the isolation and purification of pigments. Blank (14) points out that anthocyanins were shown to be amphoteric substances which build oxonium salts with acids.

Regarding the presence of more than one anthocyanin in plants, Blank (14) remarks that they are usually found as mixtures in plants. The components of these mixtures may be separated either by fractional crystallization of picrates or by the use of

chromatographic adsorption technique. Karrer et al (35) state that numerous anthocyanins change into a colorless modification, the pseudobase, in very weak, acid, neutral and specially alkaline solutions. In the last the oxonium salts can be regenerated by the use of strong acids. Blank (14) remarks that in solution the oxonium salts of anthocyanins are red in color; nevertheless, the individual types can be distinguished by the shade of color. Karrer (34) points out the fact that in aqueous and alcoholic solutions, the anthocyanins and anthocyanidins which possess two neighboring phenolic hydroxyl groups, show a color change towards violet and blue with ferric chloride. This sensitive reaction is not present with other anthocyanins and anthocyanidins. Robinson and Leon (60) have made use of the resistance of very dilute pigment solutions to ferric chloride in order to ascertain whether position 3 of the anthocyanin or of the anthocyanidin has a hydroxyl group or not. Blank (14) remarks that anthocyanins show increased blueness with increase in the hydroxyl groups and change from 3- to 3-5 sugar types. He further adds that methylation of one or more hydroxyl groups on the other hand increases the redness of these pigments. Karrer (34) has listed some of the important properties of anthocyanidins.

Schou (63) states that the anthocyanins and anthocyanidins have approximately the same absorption spectra. These compounds absorb very strongly in the investigated ranges of 2000 A° to 6200 A° and an absorption maxima is present in the visible range. He investigated various anthocyanins and anthocyanidins and found a

band at about 2700 A°. Hayashi (29) investigated the absorption spectra of flavilium salts, and the relation between light absorption and hydroxyl and sugar substitution.

Willstatter and Schmidt (97) and Willstatter et al (98) investigated the synthesis of cyanidin chloride and other anthocyanidins. Robinson (59) has approached the problem of the synthesis of anthocyanidins and anthocyanins from an entirely different angle by using condensation of ortho-hydroxy-benzaldehydes with appropriate ketones followed by ring closure. This procedure was originally adopted by Perkin (53).

Willstatter and Mallison (78) first recognized that colors of plant tissues containing anthocyanins are not due to anthocyanins themselves but also to many other factors. Blank (14) believed that color might not only be due to anthocyanins but also to the changing amounts and mixtures of them, to alteration in the pH of the cell sap, to variable ash content of the latter, to copigmentation and to the colloidal condition of certain other components of the sap.

Willstatter and Everest (76) recognized the significance of reaction of cell sap for alteration of color. Buxton et al (17) and Robinson (59) concluded that blue varieties possess a higher pH than the red varieties. He further added that these differences are too small to explain the difference in colors.

Karrer et al (35) have determined the ash content of various red and blue flower petals. They concluded that blue petals contained more ash than the red petals. This evidence is regarded as a proof that the color of red flowers is determined by the

oxonium salts, whereas that of the blue flowers is determined by alkaline salts or by those of the alkaline earths of anthocyanins. Mihailescu (50) noted no differences in the ash content of the red and blue flower petals. Lawrence et al (46) state that the pigment of the red and blue flowers is delphinidin.

Jonesco (33) pointed out the simultaneous presence of anthocyanins and tannins and their resultant additive complexes. Currey (20) made similar observations on rose petals. He found that the cause of blueing in rose petals was insufficient tannin in cell sap of the petals and that tannins are capable of forming the oxonium salts with the anthocyanin pigments. Robinson and Robinson (54) did some more work on this problem and designated it as copigmentation. Lawrence (44) carried out research on the interaction of anthocyanins and flavones. So far the pigments occurring in nature are anthoxanthins (flavones and flavonols) and tannins. He further reported that bluing phenomenon is not the result of salt formation but is evidently the result of formation of weak additive complexes, and varies according to copigment and anthocyanin concerned. Blank (14) states that since deviations in pH are usually insufficient to explain alterations in color of anthocyanin containing tissues, the condition of the pigment in solution becomes extraordinarily important together with that of copigmentation.

Robinson and Robinson (56) developed a number of quantitative tests based on the chemical behavior of anthocyanins and anthocyanidins prepared synthetically or isolated from natural sources. Using these tests they made a detailed survey on the

occurrence of anthocyanidins in the vegetable kingdom. Apart from the flowers, the other anthocyanin containing organs of the plants were also investigated. Schmid and Korperth (62) likewise investigated a series of flower extracts by means of this procedure.

The cytology of the plants with reference to anthocyanins was investigated by Herzfelder (32), and it was found that anthocyanins are found in the cell sap in the majority of cases. Crystallized anthocyanin has occasionally been detected in the cell plasma. Investigations on anthocyanins in the exines of pollens of various plants have been carried out by Bodmer (12) and Schoch-Bodmer (66).

The factors affecting formation of anthocyanins in plants have been studied by various workers. Kosaka (40) observed favorable influence of strong illumination in promoting formation of anthocyanins. Chi-Yuen-Chia (19) was able to attain a significant decrease of anthocyanin content in Amaranthus odoratus by decreasing illumination. Continuous illumination caused a discontinuation of formation of the pigment in his experiment. His results are in accord with the results obtained by Karstens (38) and Kuilman (43). These authors emphasize the fact that a photochemical as well as a dark reaction are necessary for the formation of anthocyanins.

Semmens (65) states that polarized light is able to change the anthocyanin into anthocyanidins in germinating seeds of geranium and other plants. Flint (21), Kosaka (40) and Kuilman (43) have investigated the influence of temperature on anthocyanin

formation. Since in a majority of cases the pigment was not extracted and then quantitatively determined a great many contradictory reports have appeared. Weisse (73) working with Pelargonium and Geranium spp. observed that low temperatures have an unfavorable influence on the formation of anthocyanins. Other workers (22) (41) have concluded that low temperatures have a favorable influence on the formation of anthocyanins. Steinecke (70), Vogel (71) and Woodman (101) have noticed an increase in the anthocyanin formation in the case of food starvation. Gessner and Streib (22) have investigated the formation of anthocyanins with deficiency of phosphorus, potassium and nitrogen. They observed that red coloration due to increased production of anthocyanins is increased by addition of potassium in the nutrients, whereas addition of nitrogen and phosphorus decrease the pigment content.

Specific work on the identification and isolation of anthocyanin and anthocyanidin pigments is fairly recent. The first specific work on the identification of anthocyanins of Pelargonium was done by Willstatter and Mallison (78). They isolated and analyzed the pigment in three varieties of Pelargonium. They concluded that Pelargonium zonale var. 'Meteor' has pelargonin and the bluish pink Pelargonium peltatum has the same pigment. Pelargonium zonale var. "Violet Red" was found to contain cyanin accompanied by a little pelargonin. This they say is the first case that has arisen of a variety having two anthocyanins. The authors state that variation in flower color largely depends upon the presence of other substances - acids, alkalies, salts, etc., in the cell sap.

Willstatter and Bolton (79) were the first to identify the pigments from petals of Tulipa gesneriana. They found that the scarlet red color of some varieties was due to a mixture of cyanidin diglucoside (Cyanin) and carotinoids. Robinson and Robinson (54) found that the garden tulip contained either a mixture of cyanidin and pelargonidin biosides or cyanidin biosides and delphinidin diglucoside. They further remarked that the identification of anthocyanins of tulips was much more difficult than those of most other plants.

Robinson and Robinson (54) examined 34 varieties of tulips and separated them into two groups. They stated that there was one group in which pelargonidin and cyanidin occur as 3-biosides and then, apparently with a rather sharp separation, a second group containing 'delphinidin derivatives' sometimes with cyanidin but, "They are free from pelargonidins."

The possibility of applying filter paper chromatography to the study of sap soluble plant pigments was discussed by Bate-smith (4). He pointed out that the anthocyanidins and their mono- and di-glucosides form spots well differentiated in their Rf values and give characteristic color reactions with ammonia vapor. He further points out that anthocyanidins (aglucones of anthocyanins) have to be run under standard conditions of temperature, composition of flowing solvent and the solvent in which the substance is applied to the paper. He stressed the importance of mineral acid in considerable concentration to prevent the decomposition of anthocyanins during the run. Bate-smith (9) has listed in great detail the

factors which might affect the Rf values and the precautions to be taken for getting the correct Rf. In another paper (5) the same author has described the method for the detection of leuco-anthocyanins and the chromatographic identification of the anthocyanidins from them by boiling with mineral acids. Blank (14) says that the anthocyanins usually appear as mixtures in plants and may be separated either by fractional crystallization of picrates or by the use of chromatographic adsorption technique. Karrer and Widmer (37) separated mixtures of pigments by means of chromatographic adsorption technique. Willstatter and Mallison (78) were able to isolate a mixture composed of cyanin and a little pelargonidin from a violet red variety of Pelargonium. Bate-smith (5) in another paper has improved the technique of separation of pigments and applied it to the pigments of leaves and other tissues in numerous plant species. He published the results of anthocyanin work on many important plant species including Thea sinensis and Cinnamomum sericum. He concluded that except in Rosaceae and a few Leguminoseae (which appear to contain leuco-peonidin), the leuco-anthocyanins appear to be restricted to leuco-cyanidins and leuco-delphinidins. He published a detailed list of many families in dicotyledons and a few families in monocots, Gymnosperms and Pteridophyta, indicating the plants and the anthocyanins they contain.

Many solvents have been used by various workers for running anthocyanins and anthocyanidins in the chromatographic chamber. Bate-smith (5), while discussing the merits of other solvents, has stressed the superiority of Forestal solvent supplied to him by Dr.

White of the Forestal Land, Timber and Railway Co. Harpenden, Herts. Bate-smith (5) has listed the Rf values of various anthocyanidins and anthocyanins in 3 different solvents including the Forestal solvent. Bate-smith and Westall (7) suggested maintaining a low pH of solvent during chromatography to prevent the anthocyanidins from fading out. This was achieved by them by using the upper layer of the mixture of n-butyl alcohol:2N-HCl (1:1v/v). Asen (2) has investigated the anthocyanidins and anthocyanins in Euphorbia pulcherrima by paper chromatographic methods. He has published the Rf values of anthocyanins and anthocyanidins of Euphorbia pulcherrima in three different solvents. Halevy and Asen (25) stated that the isolation and purification of anthocyanins were accomplished by column chromatography in their research with tulips. They found that the variety Pride of Haarlem contains derivatives of delphinidin, cyanidin and pelargonidin. These authors further add that a third group could be added to the classification of Robinson and Robinson (54). Robinson and Robinson (56) had stated that the tulip varieties could be grouped into two groups: 1. those varieties containing pelargonidin and cyanidin as 3-biosides, and 2. those varieties containing delphinidin derivatives, sometimes with cyanidin but free from pelargonidins. Halevy and Asen (25) add as the third group those varieties containing derivatives of delphinidin, cyanidin and pelargonidin as found in the variety Pride of Haarlem. Asen (2) identified the anthocyanins and anthocyanidins in the bracts from Euphorbia pulcherrima plants in three varieties by paper chromatographic and spectrophotometric

methods. He concluded that anthocyanins in the bracts from all the *Euphorbia* varieties examined were identical. The Rf values for the anthocyanidins studied have been listed. Halevy and Asen (25) identified the anthocyanins from the Tulip varieties Smiling Queen and Pride of Haarlem. The Rf values of the anthocyanidins from these varieties were listed in three different solvents and compared with the Rf values of authentic anthocyanidins for the purpose of identification. The anthocyanidin from the tulip variety Queen of Night was identified by Shibata (68) as delphinidin rhamnoglucoside, and Shibata and Sakai (69) identified the anthocyanidin of the variety Eclipse as cyanidin rhamnoglucoside. There is a wide range of complexity in the anthocyanidin constitution of tulips ranging from the simple one component found by Shibata (68) and Shibata and Sakai (69) to the complex multiple compounds found by Robinson and Robinson (54). Hall (26) and Beal et al (10) studied the anthocyanins of most species of the genus tulipa and found that two sub-genera differed in their anthocyanin constitution. Only pelargonidin and cyanidin were found in Leiostemones and only cyanidin and delphinidin derivatives in the Eriostemones. The anthocyanin in all cases was a pentose glycoside. The most recent work on the anthocyanins of detached petals of *Impatiens balsamina* is that of Klein and Hagen (39). They examined the anthocyanins in the detached petals of *Impatiens balsamina* and gave details of Rf valu s, etc.

The absorption spectrum has been used as a reliable guide for the identification of anthocyanins and anthocyanidins. Schou (63)

states that the anthocyanins and anthocyanidins have approximately the same absorption spectra. Bate-smith (5), Asen (2), Halevy and Asen (25) and Klein and Hagen (39) have all used the absorption spectra as a guide for the identification of anthocyanidins and anthocyanins. The absorption maximum does not differ very much for a given anthocyanin and its anthocyanidin. The absorption maxima as obtained by the above workers are as follows for the anthocyanidins: Pelargonidin, 530 mu.; Cyanidin and Peonidin, 545 mu.; Delphinidin and Malvidin, 555 mu. Asen (2) and Bate-smith (5) have given the maximum for Petunidin as 555 mu., but Halevy and Asen (25) got a higher value for the same anthocyanidin. They gave 557 mu. as the value for Petunidin. The absorption data were obtained by means of Beckman D. U. Spectrophotometer.

MATERIALS AND METHODS

The initial work was begun with geranium (Pelargonium hortorum) variety Red Irene. Red Irene is an important commercial variety of geranium having large double flowers of scarlet red color. A continual source of flowers was obtained from plants growing in the horticultural greenhouse. Freshly opened flowers of this variety were used for the determination of anthocyanidin pigments in flower petals. Preliminary work indicated that it has given two bands of pigments upon chromatographic separation. One of these was a major pigment and the other a minor one. To determine whether two anthocyanidins were peculiar to this variety alone, two additional geranium varieties, Madam Saleroi and Pink Barney were included in the

study. The flowers of Madam Saleroi are orange red in color (Plate I). It is a single flowered variety having variegated foliage. Pink Barney is a double flowered variety with clear pink colored petals. All plants were grown under similar conditions in the horticultural greenhouse.

Extraction of Pigments

Freshly opened flowers of these varieties, free from disease, insect attack or other types of damage, were used for the determination of anthocyanidins. Care was taken to use the petals as soon as possible after picking the flowers from the plants. Anthocyanidins are usually not found in nature as such, but occur as anthocyanins, in combination with sugars. For the determination of anthocyanidins, the anthocyanins must first be hydrolyzed to remove the sugar moiety. The petals were removed from two flowers of each of these varieties. Each sample, weighing 0.5 to 1.0 gram was placed in a test tube and 5 ml. of 2 N HCl was added to the tube. The petals were crushed by running a homogenizer into each tube for 2 to 4 minutes. After this treatment the petals were well crushed and formed a thin paste with HCl. Next 3 to 5 ml. of 2 N HCl was applied to the sides of each tube to wash down the crushed petals. The test tubes were placed in a steambath for hydrolysis. Complete hydrolysis is essential for the determination of anthocyanidins. The time allotted for hydrolysis is therefore very important. In the present study, time intervals ranging from thirty minutes to three hours were tested. It was found during preliminary investigations that after thirty minutes of hydrolysis, more

EXPLANATION OF PLATE I

Flowers of Geranium Varieties Under Study

Right: Var. Red Irene

Middle: Var. Madam Saleroi

Left: Pink Barney

PLATE I

than two bands were obtained upon chromatography with the variety Red Irene, while longer periods of hydrolysis resulted in the appearance of only two bands. This indicated incomplete hydrolysis in half an hour. One hour was found to be sufficient for complete hydrolysis of all varieties tested; time beyond one hour did not increase the efficiency of hydrolysis. Hydrolysis for more than two hours resulted in charring of the material, if the quantity of HCl was insufficient.

After hydrolyzing the material for one hour, the test tubes were taken from the steambath and cooled by running water on the test tubes. Three ml. of n-butyl alcohol were added to each test tube and shaken well. Butyl alcohol acts as a solvent for the anthocyanidins. The top layer of butyl alcohol containing the anthocyanidins was removed with a pipette and placed into another test tube. Sometimes the initial quantity of butyl alcohol did not prove to be sufficient for dissolving the entire quantity of anthocyanidins, and in such cases 2 to 3 ml. increments of butyl alcohol were added until there was a complete separation of anthocyanidins.

Halevy and Asen (25) have used iso-amyl alcohol instead of butyl alcohol as solvent. It was pointed out by Ahuja (1) that iso-amyl alcohol caused a brown color when heated with HCl for hydrolysis. In the present study this was confirmed. This discoloration would affect the readings obtained on the Beckman D. U. Spectrophotometer in determining the absorption maxima of anthocyanidins. N-butyl alcohol was used as a substitute for iso-amyl

alcohol in the present study. Very small quantities of iso-amyl alcohol were needed to dissolve large quantities of anthocyanidins. Concentrated solutions of anthocyanidins are necessary for chromatography on Whatman No. 1 filter paper. This is a possible explanation for the use of iso-amyl alcohol in the investigations of anthocyanidins.

Chromatographic Technique

Research (25, 2) has shown that more than one anthocyanidin may be present in the same species in a particular variety. Anthocyanidins were separated by the use of ascending method of paper chromatography. Bate-smith (9) was the first to use paper chromatography for the separation of anthocyanidins. The procedure followed in this research was similar to Bate-smith's procedure. The solution of anthocyanidins was placed at the base line of Whatman No. 1 filter paper in the form of streaks, no wider than 1 cm. The size of the paper depends upon the size of chromatographic chamber. In the present study Whatman No. 1 filter paper of the dimensions 18-1/4 x 22-1/2 inches was used and a chromatographic chamber of 2 feet in height and 12 inches in diameter. Chromatographic chamber is shown in plate II. It is a transparent glass jar of the above mentioned size with top side open. The open side was closed by a glass lid which fits tightly on the top making it air tight. To prevent leakage modeling clay was used. In the bottom of the chromatographic chamber was placed a shallow pie dish of 10 inches diameter, slightly smaller than the diameter of the chamber, in which

was placed the developing solvent. Whatman No. 1 filter paper on which the anthocyanidin solution was streaked was rolled into a cylinder and held in that position with the help of paper clips. The folded cylinder of paper with the streaked end facing downwards was lowered in the chromatographic chamber. The lower end of the paper must be in solution in the dish of the chamber. To insure uniform rising of the solvent and the pigment, it is very important that the dish and the chamber be level. The anthocyanidin solution also can be placed in spots on the filter paper. Research has shown that whether it is put in streaks or spots on the paper, it is very essential that a sufficient quantity of concentrated pigment solution is applied. If the quantity is small or the solution dilute, the different pigments will not separate clearly and there will be a tendency to form a "tail" on the chromatogram. This makes the correct determination of Rf values very difficult.

Different solvents have been suggested for separation of anthocyanidins. Bate-smith (5) recommended Forestal solvent consisting of acetic acid, hydrochloric acid and water in the proportion of 30:3:10 v/v. The Rf value obtained for a particular pigment with a particular solvent will be constant. The Rf values obtained with the Forestal solvent for the authentic anthocyanidins have been listed in table 2. In the present study, Forestal solvent alone was used. This solvent must be allowed to age for a day or two before use in the chamber. About 200 ml. of solvent was placed in the dish at the bottom of the chromatographic chamber. The filter paper was rolled and lowered into the chromatographic chamber. The Rf value

EXPLANATION OF PLATE II

Chromatographic chamber showing the filter paper folded in the form of cylinder. While in use, the chamber is covered with brown paper to keep off the light.

PLATE II

was calculated as the distance the solvent moved on the chromatogram divided by the distance the solute moved.

The time the paper is left in the chromatographic chamber is an important factor influencing the Rf value. In the present studies three different lengths of time were tried, 18 hours, 24 hours and 36 hours. During the preliminary investigations an effort was made to determine the effects of different lengths of time in chamber on Rf value.

After removal of the chromatogram from the chamber it was air dried by hanging in a hood. The heights to which the solvent and the solute rose on the paper were marked. The Rf value was calculated by taking a number of points both on solvent and solute front and calculating the averages. The Rf values of the pigments isolated were compared with those described in the literature. In a few instances pigments of known identity (Synthetic pigments) have been chromatographed on the filter paper and Rf value of the unknown compared with the Rf values of the pigment of known identity. This procedure has revealed that the synthetic anthocyanidins supplied by chemical firm were not pure and left a "tail" on chromatographic separation. This made the accurate determination of the Rf value very difficult. In the present study, therefore, identification was done by comparison with Rf values described in literature and not by running the marker of known identity.

Rf values have been found to be affected by such factors as time the paper was kept in the chamber, temperature of chamber and the type of filter paper used. A record of temperature in the

laboratory where the chromatographic chamber was placed was maintained. The average temperature during the experimental period was 80 degrees F. and varied only by ± 2.

Spectrophotometric Methods

Rf value alone cannot be taken as a reliable guide for determining the identity of unknown pigments. A further, and in fact a more reliable test is by determining the peak of maximum absorption by spectrophotometric methods. For this purpose the pigments after they were separated by chromatography were cut out from the chromatogram, eluted with 0.01 N HCl in methanol, and their absorption spectra determined with a Beckman D. U. Spectrophotometer. Both anthocyanins and anthocyanidins have definite peaks of maximum absorption in the visible spectrum. These peaks have been worked out for the authentic anthocyanidins and their values described in the literature (25). The identity of the unknown anthocyanidins was determined by comparing their peaks of maximum absorption with the absorption maxima of the authentic anthocyanidins described in the literature and reproduced here in table 2.

Of the three geranium varieties under study, the variety Pink Barney has given three pigments, one above and the other below the major pigment. The varieties Madam Saleroi and Red Irene have given two pigments, one major pigment and another minor one above the major pigment. To determine whether the minor pigments above the major one in all the three varieties is a separate pigment or is a result of incomplete hydrolysis, the minor pigment in all three

varieties was cut from the chromatogram and eluted with methanol containing 0.01 N HCl. This was hydrolyzed and again chromatographed. On chromatography it did not separate into two pigments. To insure complete hydrolysis, re-hydrolysis was done on a steam-bath as well as by heating on a hot plate to a higher temperature.

In the same way, the major band in the variety Pink Barney was cut out from the chromatogram and eluted with methanol. This was re-chromatographed to find out whether the band below the major band was a separate pigment or had resulted due to incomplete hydrolysis of the major band.

The color of the major band in all the varieties did not appear the same by visual observations. The differences of the geranium varieties under study are shown in plate I. To determine the differences in color of the major bands of the varieties in the study, all three varieties were read for their major bands in the Rapid Scanning Spectrophotometer manufactured by the American Optical Company. After chromatographic separation, the major bands in all the three varieties were cut out. An effort was made to maintain uniform concentrations in all varieties, by streaking equal quantities of pigment containing solutions on the paper. The major band cut out was held against the source of light in the reflection attachment of the Rapid Scanning Spectrophotometer.

The Rapid Scanning Spectrophotometer consists of three separate units: (1) the transmission spectrophotometer, (2) the voltage regulator, and (3) the reflection attachment (Plate IV). This apparatus has been described in great detail by Beitz (11). Before

EXPLANATION OF PLATE III

Chromatogram showing the major and minor bands in three varieties of geranium.

Left: Var. Red Irene

Middle: Var. Pink Barney

Right: Var. Madam Saleroi

PLATE III

reading the color of pigments, the machine has to be warmed up for half an hour. After this time an opaque material was inserted in place of the sample so as to cut off all light from the spectrophotometer. The up-down control was used to bring the cathode ray trace into coincidence with the zero line of the scale. The opaque material was then removed from the beam and the cathode ray trace was made to coincide with 100 percent line by using the vertical gain adjustment. The adjustment for the wave length calibration is provided by use of the didymium filter. This glass is characterized by narrow absorption bands whose wave lengths are well known. This glass was inserted in the transmittance compartment. Its spectrum was then displayed on the cathode ray tube. The horizontal gain knob and the right-left knob were used to position the spectrum so that the wave lengths of absorption maxima coincided with those given on the chart provided with the apparatus. The instrument is now in adjustment. When the major band cut out from the chromatogram was held against the source of light in the reflection unit, its reflection curve appeared on the circular screen. The points forming the curve were noted down. This curve was reproduced on a graph by plotting the points read off of the screen. The reflectance curves for the major bands in three varieties under study were plotted on the graph after calibration factors were corrected. These have been presented in plates V, VI and VII.

RESULTS

A technique for the isolation and identification of anthocyanidins

EXPLANATION OF PLATE IV

Photo of Rapid Scanning Spectrophotometer.

PLATE IV

using n-butyl alcohol was evolved. Most previous studies were conducted using dried petals with neutral lead acetate to precipitate the anthocyanins. In this study fresh petals were used and n-butyl alcohol was used as a solvent. Utilizing this technique, the anthocyanidins were isolated and separated by chromatographic methods. The Rf values of the anthocyanidins in all the three varieties under study were calculated. The Rf values were determined both by streaking the pigments on Whatman No. 1 paper and putting the same in the form of spots. The final Rf value was calculated by taking the averages of these figures. These values for the three bands of pigments in the variety Pink Barney and two bands of pigments in the varieties Red Irene and Madam Saleroi are presented in table 1. The identification of the anthocyanidins was done on the basis of comparison of Rf values worked out in the present study with those described in the literature for the authentic anthocyanidins. The Rf values for the authentic anthocyanidins described in the literature by three different authors have been presented in table 2.

A study of the chromatograms prepared with the three varieties under study showed a major band of pigment in all three varieties. This major band was the bottom band in the varieties Red Irene and Madam Saleroi which separated only in two bands (Plate III). The variety Pink Barney separated into three bands of pigments. The major band in this variety was the middle band. Besides the middle band (major band) and the top band, this variety had an additional third band below the major band which was not found in the other

two varieties.

A study of the above mentioned two tables containing the Rf values indicates that the Rf values of the major bands in all the three varieties fall within the range of Rf values described in the literature for pelargonidin. The range of Rf values for the pelargonidin is between 0.68 and 0.76. In the present study the average Rf values for the major bands of Red Irene, Madam Saleroi and Pink Barney were 0.74, 0.75 and 0.74 respectively. These values have been presented in table 3.

As these Rf values tally with the authentic Rf value of pelargonidin it seems reasonable to conclude that the major band of pigment in all the three varieties is pelargonidin.

The anthocyanidins have maximum absorption in the visible spectrum. The value of this peak which is determined by the spectrophotometric methods remains constant for a particular anthocyanidin. For the authentic anthocyanidins these values have been reported by Bate-smith (5), Halevy and Asen (25) and Asen (2), and have been reproduced in table 2. In the present study the wave lengths of maximum absorption were determined for the major bands in the three varieties under study by the spectrophotometric methods, using a Beckman D. U. Spectrophotometer. These values were read in methanol containing 0.01 N HCl. The wave lengths of maximum absorption for the major bands in all the three varieties have been presented in table I. These two tables reveal that the wave length of maximum absorption for the major bands in the three varieties comes very close to the wave length of maximum absorption for pelargonidin. The

absorption maxima for the major bands in the present study were within the range of 528 mu. to 532 mu., the values read on the Beckman D. U. Spectrophotometer remaining constant in that range. The wave length of maximum absorption for pelargonidin has been described as 530 mu. (2), (5) and (25). As the maxima for the major bands in the three varieties varies within the range of ± 2 mu., it seems safe to conclude that the anthocyanidin in the major band in all the three varieties is pelargonidin.

In the present study a new technique has been utilized for measuring the reflectances of anthocyanidins of the major bands in all the three varieties under study. Reflectance spectra were measured using a Rapid Scanning Spectrophotometer manufactured by the American Optical Company. A photograph of this machine appears on plate IV. This spectrophotometer has been described by Beitz (11). The reflectance spectra for the major bands of pigments of three varieties under study appear on plates V, VI and VII. A study of these curves shows that the curves for the varieties Red Irene and Madam Saleroi are very much similar to each other. The curve for Pink Barney is slightly different from the other two. The trend of the curves in all the three varieties under study is the same. To compare the curve on a uniform basis, a point where the curve tends to be flat was chosen. This point was at the wave length of 540 mu. The reflectance percentage at this point was compared with the reflectance percentage at the lowest point at the curves for all the three varieties under study. These ratios have been presented in table 4 (A). Similarly the ratios between the reflectance

percentages at 440 mu. and reflectance percentage on a selected point on the curve (640 mu.) were calculated for all varieties. (Table 4 (B)) A study of this table showed that the values for the ratios in table 4 (A) were quite close for all varieties under study. The values in table 4 (B) were however not as close to each other as the values in table (A). This indicated that reflectance at 640 mu. was not the same for all curves, indicating the steepness of the slope. This also showed that the trend for all the curves under study was the same and the apparent dissimilarity in curves was due to a difference in concentrations of pigments.

Besides the major bands previously described as pelargonidin, the pigment of variety Pink Barney separated into two additional minor bands, one occurring above and the other below the major band. In the varieties Red Irene and Madam Saleroi there was only one minor band besides the major band, this in both varieties was above the major band. The Rf values for the minor bands in all the varieties were determined. These values have been given in table 1.

The Rf value for the lowest minor band in the variety Pink Barney was 0.58. This value refers to the average value calculated from a number of samples. The Rf value of 0.58 falls within the range of Rf values given in table 2 for cyanidin, the range being from 0.50 to 0.60. (2), (5) and (25). As the calculated value of this band falls within this range it was suspected that the band was cyanidin. This was supported by determining the wave length of maximum absorption of this band. The wave length of maximum absorption was within the range of 528 mu. to 544 mu. The wave length

Table 1. Average Rf values and wave lengths of maximum absorption for the anthocyanidins from the acid hydrolysis of the geranium varieties under study

Compound	Rf Values in Forestal Solvent	Absorption Max. in Methanol Containing 0.01 N Hydrochloric Acid
Geranium var. Red Irene		
Band No. 1	0.87	538-540
Band No. 2 (Major band)	0.74	528-532
Geranium var. Madam Saleroi		
Band No. 1	0.88	538-540
Band No. 2 (Major band)	0.75	528-532
Geranium var. Pink Barney		
Band No. 1	0.87	538-540
Band No. 2 (Major band)	0.74	528-532
Band No. 3	0.58	528-544

Table 2. Rf values and absorption maxima of the authentic anthocyanidins

Anthocyanidin	Absorption Maxima mu.	Rf Value in Forestal Solvent	Author
Pelargonidin	530	0.68	Bate-smith (5)
	530	0.76	Halevy and Asen (25)
	530	0.74	Asen (2)
Cyanidin	545	0.50	Bate-smith (5)
	545	0.56	Halevy and Asen (25)
	545	0.60	Asen (2)
Peonidin	545	0.63	Bate-smith (5)
Delphinidin	555	0.30	Bate-smith (5)
	555	0.37	Halevy and Asen (25)
	555	0.38	Asen (2)
Petunidin	555	0.45	Bate-smith (5)
	557	0.53	Halevy and Asen (25)
Malvidin	555	0.60	Bate-smith (5)

Table 3. Rf values for the anthocyanidins in geranium varieties under study

Time in Chromatographic Chamber		Varieties		
		Red Irene	Madam Saleroi	Pink Barney
18 hours	Band No. 1	0.85	0.87	0.88
	Band No. 2	0.73	0.75	0.72
	Band No. 3	....	....	0.59
24 hours	Band No. 1	0.89	0.88	0.89
	Band No. 2	0.73	0.77	0.75
	Band No. 3	....	....	0.58
	Band No. 1	0.88	0.86	0.89
	Band No. 2	0.79	0.78	0.74
	Band No. 3	....	....	0.56
	Band No. 1	0.87	0.89	0.87
	Band No. 2	0.75	0.73	0.76
	Band No. 3	....	....	0.57
	Band No. 1	0.86	0.88	0.84
	Band No. 2	0.72	0.70	0.73
	Band No. 3	....	....	0.59
	Band No. 1	0.86	0.88	0.89
	Band No. 2	0.73	0.75	0.75
	Band No. 3	....	....	0.60
	Band No. 1	0.89	0.88	0.85
	Band No. 2	0.71	0.77	0.72
	Band No. 3	....	....	0.59
36 hours	Band No. 1	0.89	0.89	0.87
	Band No. 2	0.75	0.75	0.75
	Band No. 3	....	....	0.56
Averages	Band No. 1	0.87	0.88	0.87
	Band No. 2	0.74	0.75	0.74
	Band No. 3	....	....	0.58

Table 4. Ratios for the reflectance percentage at different points at the reflection spectra for major bands in geranium.

(A) Comparison at 440 mu. and lowest points on spectra

Reflectance Percentage at 440 mu.		Reflectance Percentage at Lowest Point on the Spectra	Ratio between 1 and 2
1		2	3
Red Irene	25	12.5	2.00
Madam Saleroi	39	20.00	1.99
Pink Barney	53	30.00	1.80

(B) Comparison at 440 mu. and 640 mu.

Reflectance Percentage at 440 mu.		Reflectance Percentage at 640 mu.	Ratio between 1 and 2
1		2	3
Red Irene	25	80	0.31
Madam Saleroi	39	91	0.42
Pink Barney	53	89	0.59

of maximum absorption for cyanidin as described in literature is 545 mu. (2), (5) and (25). As the Rf value of this band comes quite close to 545 mu., it was believed that this band was cyanidin both on the basis of Rf and wave length of maximum absorption. The difference in the wave length of maximum absorption of the determined value and the value described in literature might be attributed to the presence of some other pigment in this variety. This can be a possible explanation.

In all the three varieties under study, there was a minor band above the major band. The color of this band was pink or light red. Its Rf value and wave length of maximum absorption were determined. The average Rf value for this band for all the three varieties was 0.87. The reported values of authentic anthocyanidins described in the literature are summarized in table 2. The value of 0.87, calculated for this minor band, was too high to be considered the Rf value of any of the known authentic anthocyanidins. The wave length of maximum absorption for this band was determined. This was within the range of 538 mu. to 540 mu. This value did not tally with the wave length of maximum absorptiong iven in table 2 for any of the authentic anthocyanidins. On the basis of this evidence there is reason to believe that this band might be a new pigment not yet identified.

In order to make sure that the top pigment in all the varieties under study was not a result of incomplete hydrolysis, re-hydrolysis of this pigment was done after cutting and eluting it from the chromatogram. After re-hydrolysis the pigment was again chromatographed

On chromatography this did not separate into two bands but remained as such, indicating that hydrolysis was complete. This proves that this pigment has not resulted due to incomplete hydrolysis. It might be a new pigment not yet described in literature.

DISCUSSION

The chromatographic technique used in the present study was similar to the one outlined by Bate-smith (5). It was slightly different from the technique of Halevy and Asen (25). They have worked with dried petals using neutral lead acetate to precipitate the anthocyanins. Their procedure may have attained a higher degree of purity by eliminating the irrelevant substances. Identification of anthocyanidins was done by comparing the calculated Rf value with the values described in the literature for authentic anthocyanidins. Absorption spectra for the anthocyanidins under study were determined with a Beckman D. U. Spectrophotometer. The absorption spectra were compared with the absorption spectra of authentic anthocyanidins described in the literature. Final identification was done both on the basis of Rf value and absorption spectra.

The variety Pink Barney separated into three bands on chromatography. Varieties Red Irene and Madam Saleroi separated into two bands. All varieties under study had a major band. The Rf value of the major band was within the range of Rf values described in the literature for pelargonidin. The difference in Rf value might be due to the difference in the purity of anthocyanidins, or due to

changes in the experimental conditions.

The absorption spectra for the major bands were quite similar to the absorption spectra described in the literature for pelargonidin. In the present study the value of absorption spectra for the major band was not exactly the same as the absorption spectra for pelargonidin. It was, however, very close to it. The difference might be due to difference in purity of material or due to experimental error. As both the Rf value and the absorption spectra are quite close to the Rf value and absorption spectra for the authentic pelargonidin it seems reasonable to conclude on the basis of evidence available that the major bands in the three varieties are pelargonidin.

Besides the major band there was a minor band below the major one in the variety Pink Barney. The Rf value for this band was determined and compared with the values described in the literature for authentic anthocyanidins. Its Rf value was within the range described in the literature for cyanidin. The absorption spectra for this band was quite close to the absorption spectra described in the literature for cyanidin. The difference might be due to impurities in anthocyanidins. It might be due to copigmentation or due to difference in experimental conditions. It is very difficult to place the exact cause for this difference. It is quite likely that differences might have resulted as a cumulative effect of all these factors. The evidence based on the Rf value and absorption maxima leads us to the conclusion that the minor band below the major one in variety Pink Barney was cyanidin.

In all the varieties of geranium, there was a minor band above the major one. The Rf value of this band was determined as 0.89. This Rf value was compared with those of authentic anthocyanidins given in the literature. The Rf value of 0.89 was too high to be the value of any of the known authentic anthocyanidins described in the literature. The highest Rf in Forestal solvent for any authentic anthocyanidin described in the literature is 0.76 for pelargonidin (25). The absorption maxima for this minor band was 538-540 mu. This value did not agree with the absorption spectrum of any authentic anthocyanidin described in the literature.

To insure that this minor band was a separate compound it was re-hydrolyzed and chromatographed. On chromatography it did not separate into two pigments, indicating that hydrolysis was complete and it was a separate compound. The Rf value of this band did not agree with the Rf values described in the literature for authentic anthocyanidins. The absorption spectrum also did not agree with the known absorption spectra of authentic anthocyanidins. On the basis of this evidence, there is reason to believe that this is a new anthocyanidin not described in the literature. This band could not be identified in the present study.

A new technique used in the present study was the determination of reflectance curves for the major bands of three varieties by the Rapid Scanning Spectrophotometer. This technique has not been reported previously. The reflectance curves for the major bands of pigments in the three varieties were prepared by taking readings from the Rapid Scanning Spectrophotometer. The curves for the major bands

of three varieties looked slightly different, but still had the same trend. This difference appeared to be due to the difference in concentration of pigments. To compare these curves on a common basis, a point at 440 mu. was chosen on all curves. At this point the curves tend to be flat. The ratio of the reflectance percentage at this point and the reflectance percentage at the minimum point on the curves was calculated. (Table 4) This ration was almost the same for the three varieties.

Similar ratios were calculated by taking the reflectance percentage at 440 mu. and comparing it with 640 mu. These values for the three varieties were not as close as the values calculated with the minimum point, indicating the steepness of the slope is different.

This is a new technique and offers great promise for comparison of color for ornamental flowers. It can be a great help for determining how different two new varieties are, especially the hybrids, as far as the color is concerned. Quite often, reliance cannot be placed on visual observation. Further research is needed to perfect this technique and to explore its new uses in the field of ornamental horticulture.

SUMMARY

Three varieties of geranium (Pelargonium hortorum), Red Irene, Madam Saleroi and Pink Barney were used in the present study. The anthocyanidins from the flower petals of these varieties were extracted by the technique of Bate-smith (5), using 2N HCl and

n-butyl alcohol. Separation of anthocyanidins was done by paper chromatography. For identification, Rf values of anthocyanidins under study were compared with the Rf values of authentic anthocyanidins described in literature. Absorption spectra of the anthocyanidins in this study were compared with the absorption spectra described for authentic anthocyanidins.

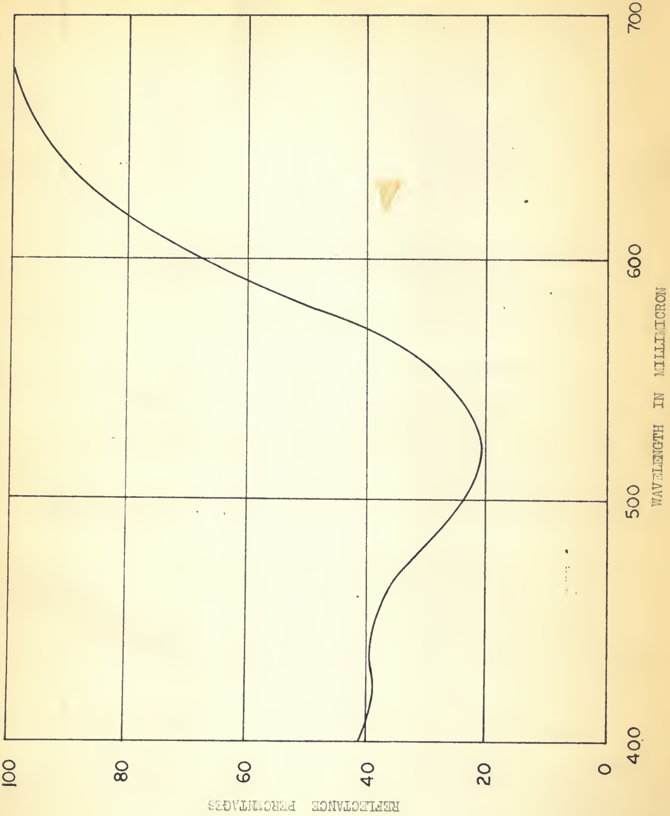
The variety Pink Barney separated on chromatography into three bands. Varieties Red Irene and Madam Saleroi separated into two bands. The major bands in all three varieties were identified as pelargonidin. The minor band below the major band in variety Pink Barney was identified as cyanidin. The minor band above the major band in all three varieties could not be identified, but appeared to be a new pigment not recorded in literature. Reflectance spectra of the major bands in all three varieties were determined by Rapid Scanning Spectrophotometer. They were quite similar to each other.

The minor band above the major band in all the three varieties needs further research for identification. The new technique of comparing the colors of pigments by reflectance curves by Rapid Scanning Spectrophotometer shows great promise in ornamental work and justifies further research.

EXPLANATION OF PLATE V

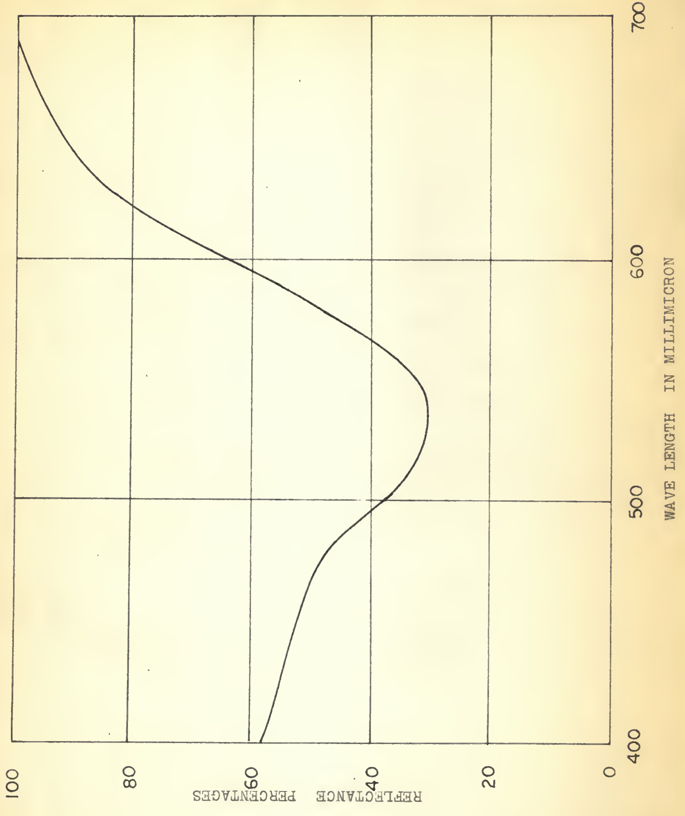
Reflectance curve for the major band of pigment in var. Madam Saleroi of geranium.

PLATE V


100
80
60
40
20
0
400
500
600
700
WAVELENGTH IN MILLIMICRON
REFLECTANCE PERCENTAGES

EXPLANATION OF PLATE VI

Reflectance curve for the major band of pigment in var. Pink Berney of geranium.



REFLECTANCE PERCENTAGES
100
80
60
40
20
0
WAVE LENGTH IN MILLIMICRON
400
500
600
700

EXPLANATION OF PLATE VII

Reflectance curve for the major band of pigment in ver. Red Irene of geranium

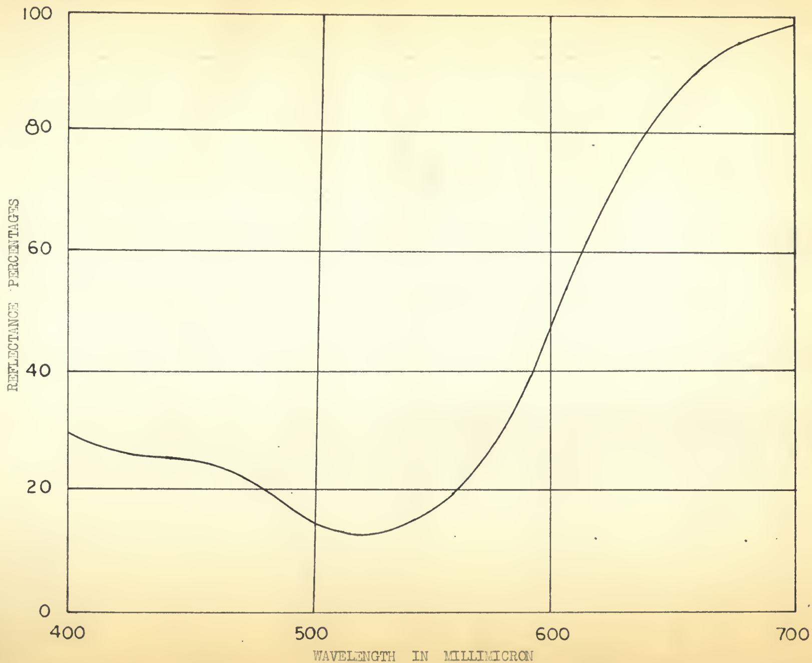

100
80
60
40
20
0
REFLECTANCE PERCENTAGES
400
500
600
700
WAVELENGTH IN MILLIMICRON

PLATE VII

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ACKNOWLEDGMENT

The author is indebted to Dr. William J. Carpenter, Associate Professor of Horticulture for suggesting the problem and for his very valuable guidance and aid in conducting this research. The author also wishes to express his thanks to Dr. Howard L. Mitchell, Head of the Department of Bio-chemistry for his technical guidance and valuable advice from time to time and for allowing the use of bio-chemistry laboratory for conducting this research. Thanks are also due to Dr. Robert P. Ealy, Head of the Department of Horticulture for giving permission to carry out this research.

IDENTIFICATION OF ANTHOCYANIDIN PIGMENTS IN THREE VARIETIES OF PELARGONIUM HORTORUM BY CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS

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AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1961

Three varieties of horticultural geranium (Pelargonium hortorum) Red Irene, Madam Saleroi and Pink Barney were selected for the identification of anthocyanidins in the flower petals. Samples of fresh petals were hydrolyzed with 2 N HCl in a steambath for one hour to remove the sugar moiety. Anthocyanidins were extracted using n-butyl alcohol as a solvent. Iso-amyl alcohol as a solvent for the anthocyanidins was found to cause discoloration during hydrolysis. In the present study, n-butyl alcohol was used as a solvent for anthocyanidins instead of iso-amyl alcohol. Separation of anthocyanidins was done by the ascending method of chromatography using Forestal solvent. Identification of anthocyanidins was done by comparing the Rf value and absorption maxima of the unknown pigments with the Rf values and absorption maxima of the authentic anthocyanidins described in the literature.

On chromatography, variety Pink Barney separated into three bands. The varieties Red Irene, and Madam Saleroi separated into two bands each. The middle band in Pink Barney and the bottom band in Red Irene and Madam Saleroi was the major band. The lowest band in Pink Barney and top bands in all three varieties were minor bands.

Rf values and absorption spectra for the major and minor bands in all varieties was determined. The Rf values of the unknown bands were determined by ascending chromatography in Forestal solvent. Absorption maxima were determined by eluting the pigments with 0.01 N HCl in methanol and reading the color with the Beckman D. U. Spectrophotometer. On the basis of Rf value and absorption maxima, the major band in all three varieties was identified as

pelargonidin. The lowest band in variety Pink Barney was identified as cyanidin. The top band in all varieties appeared to be a new pigment not described in the literature. Its Rf value and absorption maxima were determined. This band could not be identified.

A new technique used in the present study was the comparison of colors of anthocyanidin on the basis of reflectance curves prepared by reading the colors with a Rapid Scanning Spectrophotometer. This technique has not been published previously. After cutting the separated pigments from the chromatogram, the color was analyzed with a Rapid Scanning Spectrophotometer. The reflectance curves from the spectrophotometer were reproduced for an analyses. An effort was made to compare the curves on a common basis by calculating the ratio of reflectance percentages at 440 mu. and lowest point on the curves. These ratios were very close for the major bands of the three varieties. Similar ratios were calculated by taking reflectance percentages at 440 mu. and 640 mu. for all varieties. A comparison of these ratios showed similarity between the curves. The reflectance technique needs further research and investigation but shows great promise for comparison of colors.